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<p>(21) International Application Number: PCT/GB97/00390 (22) International Filing Date: 12 February 1997 (12.02.97) (30) Priority Data: 9602796.6 12 February 1996 (12.02.96) GB (71) Applicant (for all designated States except US): JOHN INNES CENTRE INNOVATIONS LIMITED [GB/GB]; Norwich Research Park, Colney Lane, Norwich NR4 7UH (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): HARBERD, Nicholas, Paul [GB/GB]; John Innes Centre, Dept. of Molecular Genetics, Colney Lane, Norwich NR4 7UJ (GB). PENG, Jinrong [CN/GB]; John Innes Centre, Dept. of Molecular Genetics, Norwich Research Park, Colney Lane, Norwich NR4 7UJ (GB). CAROL, Pierre [FR/FR]; Université Joseph Fourier, Génétique Moléculaire Végétale, B53X, F-38041 Grenoble Cédex (FR). RICHARDS, Donald, Ernest [GB/GB]; John Innes Centre, Dept. of Molecular Genetics, Norwich Research Park, Colney Lane, Norwich NR4 7UJ (GB). (74) Agents: WALTON, Sean, M. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i></p>
<p>(54) Title: NUCLEIC ACID ENCODING GAI GENE OF ARABIDOPSIS THALIANA</p>		
<p>(57) Abstract The <i>GAI</i> gene of <i>Arabidopsis thaliana</i> has been cloned, along with mutant and homologue gene sequences. Expression of such genes in plants affects characteristics of the plants including growth. <i>GAI</i> expression inhibits growth of plants, which inhibition is antagonised by gibberellin (GA). Expression of <i>gai</i> mutants confers a dwarf phenotype which is GA-insensitive. Manipulation of expression of <i>GAI</i> and <i>gai</i> genes in plants results in tall or dwarfed plants. Dwarf plants are useful in particular for reduction in crop losses resulting from lodging.</p>		

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Nucleic acid encoding GAI gene of *Arabidopsis thaliana*

This invention relates to the genetic control of growth and/or development of plants and the cloning and expression of genes involved therein. More

5 particularly, the invention relates to the cloning and expression of the GAI gene of *Arabidopsis thaliana*, and homologues from other species, and use of the genes in plants.

An understanding of the genetic mechanisms which
10 influence growth and development of plants, including flowering, provides a means for altering the characteristics of a target plant. Species for which manipulation of growth and/or development characteristics may be advantageous includes all crops,
15 with important examples being the cereals, rice and maize, probably the most agronomically important in warmer climatic zones, and wheat, barley, oats and rye in more temperate climates. Important crops for seed products are oil seed rape and canola, sugar beet,
20 maize, sunflower, soyabean and sorghum. Many crops which are harvested for their roots are, of course, grown annually from seed and the production of seed of any kind is very dependent upon the ability of the plant to flower, to be pollinated and to set seed. In
25 horticulture, control of the timing of growth and development, including flowering, is important. Horticultural plants whose flowering may be controlled include lettuce, endive and vegetable brassicas

including cabbage, broccoli and cauliflower, and carnations and geraniums. Dwarf plants on the one hand and over-size, taller plants on the other may be advantageous and/or desirable in various horticultural and agricultural contexts.

Arabidopsis thaliana is a favourite of plant geneticists as a model organism. Because it has a small, well-characterized genome, is relatively easily transformed and regenerated and has a rapid growing cycle, *Arabidopsis* is an ideal model plant in which to study growth and development and its control.

Many plant growth and developmental processes are regulated by specific members of a family of tetracyclic diterpenoid growth factors known as gibberellins (GA)¹. The *gai* mutation of *Arabidopsis* confers a dwarf phenotype and a dramatic reduction in GA-responsiveness²⁻⁹. Here we report the molecular cloning of *gai* via *Ds* transposon mutagenesis.

The phenotype conferred by the *Ds* insertion allele confirms that *gai* is a gain-of-function mutation, and that the wild-type allele (*GAI*) is dispensable^{5,6}. *GAI* encodes a novel polypeptide (*GAI*) of 532 amino acid residues, of which a 17 amino acid domain is missing in the *gai* mutant polypeptide. This result is consistent with *GAI* acting as a plant growth repressor whose activity is antagonized by GA. Though we are not to be bound by any particular theory, *gai* may repress growth constitutively because it lacks the domain that

interacts with the GA signal. Thus according to this model GA regulates plant growth by de-repression.

gai is a dominant, gain-of-function mutation, which confers a dark-green, dwarf phenotype, and interferes with GA reception or subsequent signal-transduction²⁻⁹. Dominant mutations conferring similar phenotypes are known in other plant species, including maize¹⁰⁻¹² and wheat¹³. The latter are especially important because they are the basis of the high-yielding, semi-dwarf wheat varieties of the 'green revolution'¹⁴. The increased yield of these varieties is due to an increased grain production per ear, and superior straw strength. The shorter, stronger straw greatly reduces the losses resulting from lodging, that is flattening of standing wheat plants by rain/wind. We set out to clone *gai* from *Arabidopsis* because of its importance to the understanding of GA signal-transduction, and because of the potential for use of GA-insensitivity in the development of wheat and other crops such as oil-seed rape and rice which may show improvement as great as that already seen in wheat.

According to a first aspect of the present invention there is provided a nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide with GAI function. The term "GAI function" indicates ability to influence the phenotype of a plant like the GAI gene of *Arabidopsis thaliana*. "GAI function" may be observed phenotypically in a plant as inhibition,

suppression, repression or reduction of plant growth which inhibition, suppression, repression or reduction is antagonised by GA. GAI expression tends to confer a dwarf phenotype on a plant which is antagonised by GA.

- 5 Overexpression in a plant from a nucleotide sequence encoding a polypeptide with GAI function may be used to confer a dwarf phenotype on a plant which is correctable by treatment with GA.

Also according to an aspect of the present
10 invention there is provided a nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide with ability to confer a gai mutant phenotype upon expression. gai mutant plants are dwarfed compared with wild-type, the dwarfing being GA-insensitive.

- 15 By gibberellin or GA is meant a diterpenoid molecule with the basic carbon-ring structure shown in Figure 1 and possessing biological activity, i.e. we refer to biologically active gibberellins.

Biological activity may be defined by one or more
20 of stimulation of cell elongation, leaf senescence or elicitation of the cereal aleurone α -amylase response. There are many standard assays available in the art, a positive result in any one or more of which signals a test gibberellin as biologically active^{28,29,30}.

- 25 Assays available in the art include the lettuce hypocotyl assay, cucumber hypocotyl assay, and oat first leaf assay, all of which determine biological activity on the basis of ability of an applied gibberellin to

cause elongation of the respective tissue. Preferred assays are those in which the test composition is applied to a gibberellin-deficient plant. Such preferred assays include treatment of dwarf GA-deficient *Arabidopsis* to determine growth, the dwarf pea assay, in which internode elongation is determined, the Tan-ginbozu dwarf rice assay, in which elongation of leaf sheath is determined, and the d5-maize assay, also in which elongation of leaf sheath is determined. The elongation bioassays measure the effects of general cell elongation in the respective organs and are not restricted to particular cell types.

Further available assays include the dock (*Rumex*) leaf senescence assay and the cereal aleurone α -amylase assay. Aleurone cells which surround the endosperm in grain secrete α -amylase on germination, which digests starch to produce sugars then used by the growing plant. The enzyme production is controlled by GA. Isolated aleurone cells given biologically active GA secrete α -amylase whose activity can then be assayed, for example by measurement of degradation of starch.

Structural features important for high biological activity (exhibited by GA_1 , GA_2 , GA_4 and GA_7) are a carboxyl group on C-6 of B-ring; C-19, C-10 lactone; and β -hydroxylation at C-3. β -hydroxylation at C-2 causes inactivity (exhibited by GA_8 , GA_{29} , GA_{34} and GA_{51}). *gai* mutants do not respond to GA treatment, e.g. treatment with GA_1 , GA_3 or GA_4 .

Treatment with GA is preferably by spraying with aqueous solution, for example spraying with 10^{-4} M GA₃ or GA₄ in aqueous solution, perhaps weekly or more frequently, and may be by placing droplets on plants rather than spraying. GA may be applied dissolved in an organic solvent such as ethanol or acetone, because it is more soluble in these than in water, but this is not preferred because these solvents have a tendency to damage plants. If an organic solvent is to be used, suitable formulations include 24% of 0.6, 4.0 or 300mM GA₃ or GA₄ dissolved in 80% ethanol. Plants, e.g. *Arabidopsis*, may be grown on a medium containing GA, such as tissue culture medium (GM) solidified with agar and containing supplementary GA.

15 Nucleic acid according to the present invention may have the sequence of a wild-type GAI gene of *Arabidopsis thaliana*, or be a mutant, derivative, variant or allele of the sequence provided. Preferred mutants, derivatives, variants and alleles are those which encode

20 a protein which retains a functional characteristic of the protein encoded by the wild-type gene, especially the ability for plant growth inhibition, which inhibition is antagonised by GA, or ability to confer on a plant one or more other characteristics responsive

25 to GA treatment of the plant. Other preferred mutants, derivatives, variants and alleles encode a protein which confers a *gai* mutant phenotype, that is to say reduced plant growth which reduction is insensitive to GA, i.e.

not overcome by GA treatment. Changes to a sequence, to produce a mutant, variant or derivative, may be by one or more of addition, insertion, deletion or substitution of one or more nucleotides in the nucleic acid, leading to the addition, insertion, deletion or substitution of one or more amino acids in the encoded polypeptide. Of course, changes to the nucleic acid which make no difference to the encoded amino acid sequence are included.

10 A preferred nucleotide sequence for a GAI gene is one which encodes amino acid sequence shown in Figure 4, especially a coding sequence shown in Figure 3. A preferred gai mutant lacks part or all of the 17 amino acid sequence underlined in Figure 4.

15 The present invention also provides a nucleic acid construct or vector which comprises nucleic acid with any one of the provided sequences, preferably a construct or vector from which polypeptide encoded by the nucleic acid sequence can be expressed. The
20 construct or vector is preferably suitable for transformation into a plant cell. The invention further encompasses a host cell transformed with such a construct or vector, especially a plant cell. Thus, a host cell, such as a plant cell, comprising nucleic acid
25 according to the present invention is provided. Within the cell, the nucleic acid may be incorporated within the chromosome. There may be more than one heterologous nucleotide sequence per haploid genome. This, for

example, enables increased expression of the gene product compared with endogenous levels, as discussed below.

A construct or vector comprising nucleic acid according to the present invention need not include a promoter or other regulatory sequence, particularly if the vector is to be used to introduce the nucleic acid into cells for recombination into the genome. However, in one aspect the present invention provides a nucleic acid construct comprising a *GAI* or *gai* coding sequence (which includes homologues from other than *Arabidopsis thaliana*) joined to a regulatory sequence for control of expression, the regulatory sequence being other than that naturally fused to the coding sequence and preferably of or derived from another gene.

Nucleic acid molecules and vectors according to the present invention may be as an isolate, provided isolated from their natural environment, in substantially pure or homogeneous form, or free or substantially free of nucleic acid or genes of the species of interest or origin other than the sequence encoding a polypeptide able to influence growth and/or development, which may include flowering, eg in *Arabidopsis thaliana* nucleic acid other than the *GAI* coding sequence. The term "nucleic acid isolate" encompasses wholly or partially synthetic nucleic acid.

Nucleic acid may of course be double- or single-stranded, cDNA or genomic DNA, RNA, wholly or partially

synthetic, as appropriate. Of course, where nucleic acid according to the invention includes RNA, reference to the sequence shown should be construed as reference to the RNA equivalent, with U substituted for T.

5 The present invention also encompasses the expression product of any of the nucleic acid sequences disclosed and methods of making the expression product by expression from encoding nucleic acid therefor under suitable conditions in suitable host cells. Those
10 skilled in the art are well able to construct vectors and design protocols for expression and recovery of products of recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter
15 sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor
20 Laboratory Press. Transformation procedures depend on the host used, but are well known. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA
25 into cells and gene expression, and analysis of proteins, are described in detail in *Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. Specific procedures and

vectors previously used with wide success upon plants are described by Bevan, Nucl. Acids Res. (1984) 12, 8711-8721), and Guerineau and Mullineaux, (1993) Plant transformation and expression vectors. In: Plant

5 Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148. The disclosures of Sambrook et al. and Ausubel et al. and all other documents mentioned herein are incorporated herein by reference.

10 Since the GAI amino acid sequence of *Arabidopsis* shown in Figure 4 includes 5 consecutive histidines close to its N-terminus, substantial purification of GAI or gai may be achieved using Ni-NTA resin available from QIAGEN Inc. (USA) and DIAGEN GmbH (Germany). See

15 Janknecht et al.³¹ and EP-A-0253303 and EP-A-0282042. Ni-NTA resin has high affinity for proteins with consecutive histidines close to the N- or C- terminus of the protein and so may be used to purify GAI or gai proteins from plants, plant parts or extracts or from

20 recombinant organisms such as yeast or bacteria, e.g. *E. coli*, expressing the protein.

Purified GAI protein, e.g. produced recombinantly by expression from encoding nucleic acid therefor, may be used to raise antibodies employing techniques which

25 are standard in the art. Antibodies and polypeptides comprising antigen-binding fragments of antibodies may be used in identifying homologues from other species as discussed further below.

Methods of producing antibodies include immunising a mammal (eg human, mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein or a fragment thereof. Antibodies may be obtained from immunised animals using
5 any of a variety of techniques known in the art, and might be screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage et al, 1992, Nature 357: 80-82). Antibodies may be
10 polyclonal or monoclonal.

As an alternative or supplement to immunising a mammal, antibodies with appropriate binding specificity may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, eg using
15 lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047.

Antibodies raised to a GAI, or gai, polypeptide can be used in the identification and/or isolation of
20 homologous polypeptides, and then the encoding genes. Thus, the present invention provides a method of identifying or isolating a polypeptide with GAI function or ability to confer a gai mutant phenotype, comprising screening candidate polypeptides with a polypeptide
25 comprising the antigen-binding domain of an antibody (for example whole antibody or a fragment thereof) which is able to bind an Arabidopsis GAI or gai polypeptide, or preferably has binding specificity for such a

polypeptide, such as having the amino acid sequence shown in Figure 4.

Candidate polypeptides for screening may for instance be the products of an expression library
5 created using nucleic acid derived from a plant of interest, or may be the product of a purification process from a natural source.

A polypeptide found to bind the antibody may be isolated and then may be subject to amino acid
10 sequencing. Any suitable technique may be used to sequence the polypeptide either wholly or partially (for instance a fragment of the polypeptide may be sequenced). Amino acid sequence information may be used in obtaining nucleic acid encoding the polypeptide, for
15 instance by designing one or more oligonucleotides (e.g. a degenerate pool of oligonucleotides) for use as probes or primers in hybridisation to candidate nucleic acid, as discussed further below.

A further aspect of the present invention provides
20 a method of identifying and cloning GAI homologues from plant species other than *Arabidopsis thaliana* which method employs a nucleotide sequence derived from that shown in Figure 3. Sequences derived from these may themselves be used in identifying and in cloning other
25 sequences. The nucleotide sequence information provided herein, or any part thereof, may be used in a data-base search to find homologous sequences, expression products of which can be tested for GAI function. Alternatively,

nucleic acid libraries may be screened using techniques well known to those skilled in the art and homologous sequences thereby identified then tested.

For instance, the present invention also provides a
5 method of identifying and/or isolating a GAI or gai
homologue gene, comprising probing candidate (or
"target") nucleic acid with nucleic acid which encodes a
polypeptide with GAI function or a fragment or mutant,
derivative or allele thereof. The candidate nucleic
10 acid (which may be, for instance, cDNA or genomic DNA)
may be derived from any cell or organism which may
contain or is suspected of containing nucleic acid
encoding such a homologue.

In a preferred embodiment of this aspect of the
15 present invention, the nucleic acid used for probing of
candidate nucleic acid encodes an amino acid sequence
shown in Figure 4, a sequence complementary to a coding
sequence, or a fragment of any of these, most preferably
comprising a nucleotide sequence shown in Figure 3.

20 Alternatively, as discussed, a probe may be
designed using amino acid sequence information obtained
by sequencing a polypeptide identified as being able to
be bound by an antigen-binding domain of an antibody
which is able to bind a GAI or gai polypeptide such as
25 one with the amino acid sequence shown in Figure 4.

Preferred conditions for probing are those which
are stringent enough for there to be a simple pattern
with a small number of hybridizations identified as

positive which can be investigated further. It is well known in the art to increase stringency of hybridisation gradually until only a few positive clones remain.

As an alternative to probing, though still
5 employing nucleic acid hybridisation, oligonucleotides designed to amplify DNA sequences from GAI genes may be used in PCR or other methods involving amplification of nucleic acid, using routine procedures. See for instance "PCR protocols; A Guide to Methods and
10 Applications", Eds. Innis et al, 1990, Academic Press, New York.

Preferred amino acid sequences suitable for use in the design of probes or PCR primers are sequences conserved (completely, substantially or partly) between
15 GAI genes.

On the basis of amino acid sequence information, oligonucleotide probes or primers may be designed, taking into account the degeneracy of the genetic code, and, where appropriate, codon usage of the organism from
20 the candidate nucleic acid is derived.

The present invention also extends to nucleic acid encoding a GAI homologue obtained using a nucleotide sequence derived from that shown in Figure 3.

Also included within the scope of the present
25 invention are nucleic acid molecules which encode amino acid sequences which are homologues of the polypeptide encoded by GAI of *Arabidopsis thaliana*. A homologue may be from a species other than *Arabidopsis thaliana*.

Homology may be at the nucleotide sequence and/or amino acid sequence level. Preferably, the nucleic acid and/or amino acid sequence shares homology with the sequence encoded by the nucleotide sequence of Figure 3, preferably at least about 50%, or 60%, or 70%, or 80% homology, most preferably at least 90% or 95% homology. Nucleic acid encoding such a polypeptide may preferably share with the *Arabidopsis thaliana* GAI gene the ability to confer a particular phenotype on expression in a plant, preferably a phenotype which is GA responsive (i.e. there is a change in a characteristic of the plant on treatment with GA), such as the ability to inhibit plant growth where the inhibition is antagonised by GA. As noted, GAI expression in a plant may affect one or more other characteristics of the plant. A preferred characteristic that may be shared with the *Arabidopsis thaliana* GAI gene is the ability to complement a GAI null mutant phenotype in a plant such as *Arabidopsis thaliana*, such phenotype being resistance to the dwarfing effect of paclobutrazol.

Some preferred embodiments of polypeptides according to the present invention (encoded by nucleic acid embodiments according to the present invention) include the 17 amino acid sequence which is underlined in Figure 4 or a contiguous sequence of amino acids residues with at least about 10 residues with similarity or identity with the respective corresponding residue (in terms of position) in 17 amino acids which are

underlined in Figure 4, more preferably, 11, 12, 13, 14, 15, 16 or 17 such residues.

As is well-understood, homology at the amino acid level is generally in terms of amino acid similarity or
5 identity. Similarity allows for "conservative variation", i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for
10 aspartic acid, or glutamine for asparagine. Similarity may be as defined and determined by the TBLASTN program, of Altschul et al. (1990) *J. Mol. Biol.* 215: 403-10, which is in standard use in the art. Homology may be over the full-length of the GAI sequence of Figure 4, or
15 may more preferably be over a contiguous sequence of 17 amino acids, compared with the 17 amino acids underlined in Figure 4, or a longer sequence, e.g. about 20, 25, 30, 40, 50 or more amino acids, compared with the amino acid sequence of Figure 4 and preferably including the
20 underlined 17 amino acids.

At the nucleic acid level, homology may be over the full-length or more preferably by comparison with the 51 nucleotide coding sequence within the sequence of Figure 3 and encoding the 17 amino acid sequence underlined in
25 Figure 4, or a longer sequence, e.g. about, 60, 70, 80, 90, 100, 120, 150 or more nucleotides and preferably including the 51 nucleotide of Figure 3 which encodes the underlined 17 amino acid sequence of Figure 4.

Homologues to *gai* mutants are also provided by the present invention. These may be mutants where the wild-type includes the 17 amino acids underlined in Figure 4, or a contiguous sequence of 17 amino acids with at least about 10 (more preferably, 11, 12, 13, 14, 15, 16 or 17) which have similarity or identity with the corresponding residue in the 17 amino acid sequence underlined in Figure 4, but the mutant does not. Nucleic acid encoding such mutant polypeptides may on expression in a plant confer a phenotype which is insensitive or unresponsive to treatment of the plant with GA, that is a mutant phenotype which is not overcome or there is no reversion to wild-type phenotype on treatment of the plant with GA (though there may be some response in the plant on provision or depletion of GA).

A further aspect of the present invention provides a nucleic acid isolate having a nucleotide sequence encoding a polypeptide which includes an amino acid sequence which is a mutant, allele, derivative or variant sequence of the *GAI* amino acid sequence of the species *Arabidopsis thaliana* shown in Figure 4, or is a homologue of another species or a mutant, allele, derivative or variant thereof, wherein said mutant, allele, derivative, variant or homologue differs from the amino acid sequence shown in Figure 4 by way of insertion, deletion, addition and/or substitution of one or more amino acids, as obtainable by producing transgenic plants by transforming plants which have a

GAI null mutant phenotype, which phenotype is resistance to the dwarfing effect of paclobutrazol, with test nucleic acid, causing or allowing expression from test nucleic acid within the transgenic plants, screening the 5 transgenic plants for those exhibiting complementation of the GAI null mutant phenotype to identify test nucleic acid able to complement the GAI null mutant, deleting from nucleic acid so identified as being able to complement the GAI null mutant a nucleotide sequence 10 encoding the 17 amino acid sequence underlined in Figure 4 or a contiguous 17 amino acid sequence in which at least 10 residues have similarity or identity with the respective amino acid in the corresponding position in the 17 amino acid sequence underlined in Figure 4, more 15 preferably 11, 12, 13, 14, 15, 16 or 17.

GAI and gai gene homologues may be identified from economically important monocotyledonous crop plants such as wheat, rice and maize. Although genes encoding the same protein in monocotyledonous and dicotyledonous 20 plants show relatively little homology at the nucleotide level, amino acid sequences are conserved.

In public sequence databases we recently identified several EST sequences that were obtained in random sequencing programmes and share homology with GAI. Table 25 2 gives details, showing that homologous sequences have been found in various species, including *Zea Mays* (maize), *O. Sativa* (rice), and *Brassica napus* (rape). By sequencing, study of expression patterns and

examining the effect of altering their expression, GAI gene homologues, carrying out a similar function in other plants, are obtainable. Of course, novel uses and mutants, derivatives and alleles of these sequences are
5 included within the scope of the various aspects of the present invention in the same terms as discussed above for the *Arabidopsis thaliana* gene.

A cell containing nucleic acid of the present invention represents a further aspect of the invention,
10 particularly a plant cell, or a bacterial cell.

The cell may comprise the nucleic acid encoding the enzyme by virtue of introduction into the cell or an ancestor thereof of the nucleic acid, e.g. by transformation using any suitable technique available to
15 those skilled in the art.

Also according to the invention there is provided a plant cell having incorporated into its genome nucleic acid as disclosed. The present invention also provides a plant comprising such a plant cell.

20 Also according to the invention there is provided a plant cell having incorporated into its genome a sequence of nucleotides as provided by the present invention, under operative control of a regulatory sequence for control of expression. A further aspect of
25 the present invention provides a method of making such a plant cell involving introduction of a vector comprising the sequence of nucleotides into a plant cell and causing or allowing recombination between the vector and

the plant cell genome to introduce the sequence of nucleotides into the genome.

A plant according to the present invention may be one which does not breed true in one or more properties.

5 Plant varieties may be excluded, particularly registrable plant varieties according to Plant Breeders' Rights. It is noted that a plant need not be considered a "plant variety" simply because it contains stably within its genome a transgene, introduced into a cell of

10 the plant or an ancestor thereof.

In addition to a plant, the present invention provides any clone of such a plant, seed, selfed or hybrid progeny and descendants, and any part of any of these, such as cuttings, seed. The invention provides

15 any plant propagule, that is any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on. Also encompassed by the invention is a plant which is a sexually or asexually propagated off-spring, clone or descendant of

20 such a plant, or any part or propagule of said plant, off-spring, clone or descendant.

The invention further provides a method of influencing the characteristics of a plant comprising expression of a heterologous GAI or gai gene sequence

25 (or mutant, allele, derivative or homologue thereof, as discussed) within cells of the plant. The term "heterologous" indicates that the gene/sequence of nucleotides in question have been introduced into said

cells of the plant, or an ancestor thereof, using genetic engineering, that is to say by human intervention, which may comprise transformation. The gene may be on an extra-genomic vector or incorporated, preferably stably, into the genome. The heterologous gene may replace an endogenous equivalent gene, ie one which normally performs the same or a similar function in control of growth and/or development, or the inserted sequence may be additional to an endogenous gene. An advantage of introduction of a heterologous gene is the ability to place expression of the gene under the control of a promoter of choice, in order to be able to influence gene expression, and therefore growth and/or development of the plant according to preference. Furthermore, mutants and derivatives of the wild-type gene may be used in place of the endogenous gene. The inserted gene may be foreign or exogenous to the host cell, e.g. of another plant species.

The principal characteristic which may be altered using the present invention is growth.

According to the model of the GAI gene as a growth repressor, under-expression of the gene may be used to promote growth, at least in plants which have only one endogenous gene conferring GAI function (not for example *Arabidopsis* which has endogenous homologues which would compensate). This may involve use of anti-sense or sense regulation. Taller plants may be made by knocking out GAI or the relevant homologous gene in the plant of

interest. Plants may be made which are resistant to compounds which inhibit GA biosynthesis, such as paclobutrazol, for instance to allow use of a GA biosynthesis inhibitor to keep weeds dwarf but let crop
5 plants grow tall.

Over-expression of a GAI gene may lead to a dwarf plant which is correctable by treatment with GA, as predicted by the GAI repression model.

Since *gai* mutant genes are dominant on phenotype,
10 they may be used to make GA-insensitive dwarf plants. This may be applied for example to any transformable crop-plant, tree or fruit-tree species. It may provide higher yield/reduced lodging like *Rht* wheat. In rice this may provide GA-insensitive rice resistant to the
15 Bakane disease, which is a problem in Japan and elsewhere. Dwarf ornamentals may be of value for the horticulture and cut-flower markets. Sequence manipulation may provide for varying degrees of severity of dwarfing, GA-insensitive phenotype, allowing
20 tailoring of the degree of severity to the needs of each crop-plant or the wishes of the manipulator. Over-expression of *gai*-mutant sequences is potentially the most useful.

A second characteristic that may be altered is
25 plant development, for instance flowering. In some plants, and in certain environmental conditions, a GA signal is required for floral induction. For example, GA-deficient mutant *Arabidopsis* plants grown under short

day conditions will do not flower unless treated with GA: these plants do flower normally when grown under long day conditions. *Arabidopsis gai* mutant plants show delayed flowering under short day conditions: severe mutants may not flower at all. Thus, for instance by GAI or *gai* gene expression or over-expression, plants may be produced which remain vegetative until given GA treatment to induce flowering. This may be useful in horticultural contexts or for spinach, lettuce and other crops where suppression of bolting is desirable.

The nucleic acid according to the invention may be placed under the control of an externally inducible gene promoter to place the GAI or *gai* coding sequence under the control of the user.

The term "inducible" as applied to a promoter is well understood by those skilled in the art. In essence, expression under the control of an inducible promoter is "switched on" or increased in response to an applied stimulus. The nature of the stimulus varies between promoters. Some inducible promoters cause little or undetectable levels of expression (or no expression) in the absence of the appropriate stimulus. Other inducible promoters cause detectable constitutive expression in the absence of the stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible promoter is increased in the presence of the correct stimulus. The preferable situation is where the level of expression increases

upon application of the relevant stimulus by an amount effective to alter a phenotypic characteristic. Thus an inducible (or "switchable") promoter may be used which causes a basic level of expression in the absence of the stimulus which level is too low to bring about a desired phenotype (and may in fact be zero). Upon application of the stimulus, expression is increased (or switched on) to a level which brings about the desired phenotype.

Suitable promoters include the Cauliflower Mosaic Virus 35S (CaMV 35S) gene promoter that is expressed at a high level in virtually all plant tissues (Benfey et al, 1990a and 1990b); the maize glutathione-S-transferase isoform II (GST-II-27) gene promoter which is activated in response to application of exogenous safener (WO93/01294, ICI Ltd); the cauliflower meristem promoter that is expressed in the vegetative apical meristem as well as several well localised positions in the plant body, eg inner phloem, flower primordia, branching points in root and shoot (Medford, 1992; Medford et al, 1991) and the *Arabidopsis thaliana* LEAFY promoter that is expressed very early in flower development (Weigel et al, 1992).

The GST-II-27 gene promoter has been shown to be induced by certain chemical compounds which can be applied to growing plants. The promoter is functional in both monocotyledons and dicotyledons. It can therefore be used to control gene expression in a variety of genetically modified plants, including field

crops such as canola, sunflower, tobacco, sugarbeet, cotton; cereals such as wheat, barley, rice, maize, sorghum; fruit such as tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, and melons; and
5 vegetables such as carrot, lettuce, cabbage and onion. The GST-II-27 promoter is also suitable for use in a variety of tissues, including roots, leaves, stems and reproductive tissues.

Accordingly, the present invention provides in a
10 further aspect a gene construct comprising an inducible promoter operatively linked to a nucleotide sequence provided by the present invention, such as the GAI gene of *Arabidopsis thaliana*, a homologue from another plant species or any mutant, derivative or allele thereof.
15 This enables control of expression of the gene. The invention also provides plants transformed with said gene construct and methods comprising introduction of such a construct into a plant cell and/or induction of expression of a construct within a plant cell, by
20 application of a suitable stimulus, an effective exogenous inducer. The promoter may be the GST-II-27 gene promoter or any other inducible plant promoter.

When introducing a chosen gene construct into a cell, certain considerations must be taken into account,
25 well known to those skilled in the art. The nucleic acid to be inserted should be assembled within a construct which contains effective regulatory elements which will drive transcription. There must be available

a method of transporting the construct into the cell. Once the construct is within the cell membrane, integration into the endogenous chromosomal material either will or will not occur. Finally, as far as
5 plants are concerned the target cell type must be such that cells can be regenerated into whole plants.

Selectable genetic markers may be used consisting of chimaeric genes that confer selectable phenotypes such as resistance to antibiotics such as kanamycin,
10 hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones and glyphosate.

An aspect of the present invention is the use of nucleic acid according to the invention in the
15 production of a transgenic plant.

A further aspect provides a method including introducing the nucleic acid into a plant cell and causing or allowing incorporation of the nucleic acid into the genome of the cell.

20 Any appropriate method of plant transformation may be used to generate plant cells comprising nucleic acid in accordance with the present invention. Following transformation, plants may be regenerated from transformed plant cells and tissue.

25 Successfully transformed cells and/or plants, i.e. with the construct incorporated into their genome, may be selected following introduction of the nucleic acid into plant cells, optionally followed by regeneration

into a plant, e.g. using one or more marker genes such as antibiotic resistance (see above).

Plants transformed with the DNA segment containing the sequence may be produced by standard techniques
5 which are already known for the genetic manipulation of plants. DNA can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by *Agrobacterium* exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR
10 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966, Green et al. (1987) *Plant Tissue and Cell Culture*, Academic Press), electroporation (EP 290395, WO
15 8706614 Gelvin Debeyser - see attached) other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611), liposome mediated DNA uptake (e.g. Freeman et al. *Plant Cell Physiol.* 29: 1353 (1984)), or the vortexing method (e.g. Kindle, *PNAS U.S.A.* 87: 1228 (1990d). Physical
20 methods for the transformation of plant cells are reviewed in Oard, 1991, *Biotech. Adv.* 9: 1-11.

Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous species. Recently, there has been substantial progress
25 towards the routine production of stable, fertile transgenic plants in almost all economically relevant monocot plants (Toriyama, et al. (1988) *Bio/Technology* 6, 1072-1074; Zhang, et al. (1988) *Plant Cell Rep.* 7,

379-384; Zhang, et al. (1988) *Theor Appl Genet* 76, 835-840; Shimamoto, et al. (1989) *Nature* 338, 274-276; Datta, et al. (1990) *Bio/Technology* 8, 736-740; Christou, et al. (1991) *Bio/Technology* 9, 957-962; Peng, et al. (1991) International Rice Research Institute, Manila, Philippines 563-574; Cao, et al. (1992) *Plant Cell Rep.* 11, 585-591; Li, et al. (1993) *Plant Cell Rep.* 12, 250-255; Rathore, et al. (1993) *Plant Molecular Biology* 21, 871-884; Fromm, et al. (1990) *Bio/Technology* 8, 833-839; Gordon-Kamm, et al. (1990) *Plant Cell* 2, 603-618; D'Halluin, et al. (1992) *Plant Cell* 4, 1495-1505; Walters, et al. (1992) *Plant Molecular Biology* 18, 189-200; Koziel, et al. (1993) *Biotechnology* 11, 194-200; Vasil, I. K. (1994) *Plant Molecular Biology* 25, 925-937; Weeks, et al. (1993) *Plant Physiology* 102, 1077-1084; Somers, et al. (1992) *Bio/Technology* 10, 1589-1594; WO92/14828). In particular, *Agrobacterium* mediated transformation is now emerging also as an highly efficient transformation method in monocots (Hiei et al. (1994) *The Plant Journal* 6, 271-282).

The generation of fertile transgenic plants has been achieved in the cereals rice, maize, wheat, oat, and barley (reviewed in Shimamoto, K. (1994) *Current Opinion in Biotechnology* 5, 158-162.; Vasil, et al. (1992) *Bio/Technology* 10, 667-674; Vain et al., 1995, *Biotechnology Advances* 13 (4): 653-671; Vasil, 1996, *Nature Biotechnology* 14 page 702).

Microprojectile bombardment, electroporation and

direct DNA uptake are preferred where *Agrobacterium* is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, eg
5 bombardment with *Agrobacterium* coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with *Agrobacterium* (EP-A-486233).

Brassica napus transformation is described in
10 Moloney et al. (1989) *Plant Cell Reports* 8: 238-242.

Following transformation, a plant may be regenerated, e.g. from single cells, callus tissue or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues and
15 organs of the plant. Available techniques are reviewed in Vasil et al., *Cell Culture and Somatic Cell Genetics of Plants, Vol I, II and III, Laboratory Procedures and Their Applications*, Academic Press, 1984, and Weissbach and Weissbach, *Methods for Plant Molecular Biology*,
20 Academic Press, 1989.

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the
25 invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not

essential to or a limitation of the invention, nor is the choice of technique for plant regeneration.

In the present invention, over-expression may be achieved by introduction of the nucleotide sequence in a sense orientation. Thus, the present invention provides a method of influencing a characteristic of a plant, the method comprising causing or allowing expression of nucleic acid according to the invention from that nucleic acid within cells of the plant.

Under-expression of the gene product polypeptide may be achieved using anti-sense technology or "sense regulation". The use of anti-sense genes or partial gene sequences to down-regulate gene expression is now well-established. DNA is placed under the control of a promoter such that transcription of the "anti-sense" strand of the DNA yields RNA which is complementary to normal mRNA transcribed from the "sense" strand of the target gene. For double-stranded DNA this is achieved by placing a coding sequence or a fragment thereof in a "reverse orientation" under the control of a promoter. The complementary anti-sense RNA sequence is thought then to bind with mRNA to form a duplex, inhibiting translation of the endogenous mRNA from the target gene into protein. Whether or not this is the actual mode of action is still uncertain. However, it is established fact that the technique works. See, for example, Rothstein et al, 1987; Smith et al, (1988) Nature 334, 724-726; Zhang et al, (1992) The Plant Cell 4, 1575-1588,

English et al., (1996) *The Plant Cell* 8, 179-188.

Antisense technology is also reviewed in reviewed in Bourque, (1995), *Plant Science* 105, 125-149, and Flavell, (1994) *PNAS USA* 91, 3490-3496.

5 The complete sequence corresponding to the coding sequence in reverse orientation need not be used. For example fragments of sufficient length may be used. It is a routine matter for the person skilled in the art to screen fragments of various sizes and from various parts
10 of the coding sequence to optimise the level of anti-sense inhibition. It may be advantageous to include the initiating methionine ATG codon, and perhaps one or more nucleotides upstream of the initiating codon. A further possibility is to target a regulatory sequence of a
15 gene, e.g. a sequence that is characteristic of one or more genes in one or more pathogens against which resistance is desired. A suitable fragment may have at least about 14-23 nucleotides, e.g. about 15, 16 or 17, or more, at least about 25, at least about 30, at least
20 about 40, at least about 50, or more. Such fragments in the sense orientation may be used in co-suppression (see below).

Total complementarity of sequence is not essential, though may be preferred. One or more nucleotides may
25 differ in the anti-sense construct from the target gene. It may be preferred for there to be sufficient homology for the respective anti-sense and sense RNA molecules to hybridise, particularly under the conditions existing in

a plant cell.

Thus, the present invention also provides a method of influencing a characteristic of a plant, the method comprising causing or allowing anti-sense transcription
5 from nucleic acid according to the invention within cells of the plant.

When additional copies of the target gene are inserted in sense, that is the same, orientation as the target gene, a range of phenotypes is produced which
10 includes individuals where over-expression occurs and some where under-expression of protein from the target gene occurs. When the inserted gene is only part of the endogenous gene the number of under-expressing individuals in the transgenic population increases. The
15 mechanism by which sense regulation occurs, particularly down-regulation, is not well-understood. However, this technique is also well-reported in scientific and patent literature and is used routinely for gene control. See, for example, See, for example, van der Krol et al.,
20 (1990) *The Plant Cell* 2, 291-299; Napoli et al., (1990) *The Plant Cell* 2, 279-289; Zhang et al., (1992) *The Plant Cell* 4, 1575-1588, and US-A-5,231,020.

Thus, the present invention also provides a method of influencing a characteristic of a plant, the method
25 comprising causing or allowing expression from nucleic acid according to the invention within cells of the plant. This may be used to influence growth.

Aspects and embodiments of the present invention will now be illustrated, by way of example, with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

The following Figures are included herein:

Figure 1: The basic carbon-ring structure of gibberellins.

Figure 2: The *gai-t6* line contains a transposed *Ds* which interrupts a transcribed gene.

Figure 2a: Plants shown are (left to right) homozygous for *GAI*, *gai* and *gai-t6*. *GAI* and *gai-t6* plants are indistinguishable.

Figure 2b: DNA gel-blot hybridization using a *Ds* probe. DNA in the *GAI* lane lacks *Ds*. The *gai* lane contains DNA from plants homozygous for *gai* and for T-DNA A264⁵, which contains *Ds* (18.0 kb *EcoRI* fragment). The *gai-t6* lane contains DNA from plants homozygous for A264 and for a transposed *Ds* (15.5 kb fragment).

Figure 2c: DNA gel-blot hybridization using a radiolabelled *GAI* cDNA probe. The cDNA hybridizes with a 5.1 kb *BclI* fragment in DNA from *GAI* and *gai*, replaced in *gai-t6* by fragments of 6.4 and 2.8 kb. Since *BclI* cuts once within *Ds*, the *Ds* insertion is flanked on either side by the gene (*GAI*) encoding the cDNA. The fainter hybridization at 1.7 kb is one of several seen

on longer exposure and identifies a sequence related to GAI.

Figure 3: A nucleotide sequence of a GAI gene encoding a polypeptide with GAI function.

5 Figure 4: Primary structure of GAI and gai proteins. The amino acid sequence predicted from the genomic DNA sequence of GAI is shown. The 17 amino acid segment deleted in gai is shown in bold face and double-underlined.

10 Figure 5: De-repression model for plant growth regulation by GA.

Figure 6: Nucleotide and encoded amino acid sequences of gai-derivative alleles.

Figure 6a: Nucleotide sequence of gai-d1.
15 Figure 6b: Amino acid sequence of gai-d1.
Figure 6c: Nucleotide sequence of gai-d2.
Figure 6d: Amino acid sequence of gai-d2.
Figure 6e: Nucleotide sequence of gai-d5.
Figure 6f: Amino acid sequence of gai-d5.
20 Figure 6g: Nucleotide sequence of gai-d7.
Figure 6h: Amino acid sequence of gai-d7.

EXAMPLE 1

Cloning of and characterisation of GAI and gai genes

25 gai maps to chromosome 1² of Arabidopsis, approximately 11 cM from a T-DNA insertion carrying a Ds transposon^{5,15}. Genetic analyses suggested that loss-of-function alleles confer a tall phenotype

indistinguishable from that conferred by the wild-type allele (*GAI*)^{5,6}. We attempted to clone *GAI* via insertional mutagenesis, exploiting the tendency of *Ds* to transpose preferentially to linked sites^{16,17}.

5 Plant lines homozygous for A264 and *gai*, containing a transgene (Δ NaeI-sAc(GUS)-1) expressing *Ac* transposase were constructed. Plants homozygous for a putative *Ds* insertion allele, which we designated *gai-t6*, were isolated from this material as follows⁵. The material
10 was bulked up, by self-pollination, over several generations. During this bulking, searches were made for plants which had stem branches more elongated than expected for a *gai* homozygote. Seeds obtained from self-pollination of such branches were planted out for
15 closer examination. The progeny of one such branch segregated plants, at a frequency of approximately one quarter, displaying a tall phenotype indistinguishable from that conferred by *GAI* (Figure 2a). These plants were homozygous for a new *gai* allele, which we
20 designated *gai-t6*.

DNA gel-blot experiments revealed that *gai-t6* contains a transposed *Ds* (Figure 2b), inserted within a region (approximately 200kb) of chromosome 1 known to contain *GAI* (data not shown). Genomic DNA preparation
25 and gel-blot hybridizations were performed as described⁵. *EcoRI* digests were hybridized with the *Ds* probe (radiolabelled 3.4 kb *XhoI*-*BamHI* subfragment of *Ac*). *gai-t6* has lost (Δ NaeI-sAc(GUS)-1) via genetic

segregation.

Further experiments showed that the transposed *Ds* interrupts the transcribed region of a gene (*GAI*), and that the *Arabidopsis* genome contains at least one additional gene sharing significant sequence homology with *GAI* (Figure 2c). A radiolabelled IPCR fragment containing genomic DNA adjacent to the 3' end of the transposed *Ds* in *gai-t6* was isolated as previously described²⁴. It was necessary to use considerable caution in the use of this probe since it was potentially contaminated with sequence derived from the T-DNA 3' of the *Ds* in A264 (which is still present in the *gai-t6* line): However, the fact that the probe hybridized with DNA from plants lacking any T-DNA insertion indicated that it was useful for the purposes of cloning the region of genomic DNA into which the transposed *Ds* in *gai-t6* had inserted. This probe was shown to hybridize to genomic DNA cosmid clones previously identified as being likely to contain *GAI* by map-based cloning. One of these cosmids was used to identify, by hybridization, clones from a cDNA library made from mRNA isolated from aerial plant parts (*Arabidopsis*). These cDNAs were classified according to their hybridization to genomic DNA from *GAI*, *gai* and *gai-t6*. Some of these clones hybridized weakly fragments containing *GAI* (as defined by the alteration in fragment size caused by the *Ds* insertion in *gai-t6*), but more strongly to other, related sequences. These

cdNAs are presumably derived from mRNAs transcribed from genes related in sequence to *GAI*, but not from *GAI* itself, and were put to one side for future investigations. One cdNA, pPC1, hybridized strongly to *GAI*, and less strongly to the fragments containing sequence related to *GAI*. The DNA sequence of part this cdNA was identical with approximately 150 bp of genomic DNA flanking the *Ds* insertion in *gai-t6*.

Reversion analysis showed that excision of *Ds* from *gai-t6* was associated with restoration of a dominant dwarf phenotype.

The DNA sequences of two overlapping *GAI* cdNAs revealed an open reading frame (ORF) encoding a protein (*GAI*) of 532 amino acid residues. DNA fragments containing this ORF were amplified from *GAI* and *gai* genomic DNA. Oligonucleotide primers derived from the DNA sequences of overlapping cdNAs pPC1 and pPC2 were used to amplify, via PCR, 1.7 kb fragments from *GAI* and *gai* genomic DNA. The sequences of the primers used were:

Primer N6: 5'TAG AAG TGG TAG TGG3';

Primer AT1: 5'ACC ATG AGA CCA GCC G3'.

The sequence of primer AT1 differs by one base from the sequence of the genomic and c-DNA clones. The primer was synthesized very early in the sequencing project, before the final corrected version of the sequence was available.

The DNA sequences of fragments from duplicate

amplifications were determined, thus avoiding errors introduced by PCR.

The GAI genomic sequence was almost identical with that of the overlapping cDNAs. There were three
5 nucleotide substitutions that could be due to differences between ecotypes and which do not alter the predicted amino acid sequence of GAI. The sequences of these genomic fragments revealed that the ORF is not interrupted by introns (Figure 3).

10 The *Ds* insertion in *gai-t6* is located between the Glu¹⁸² and Asn¹⁸³ codons (Figure 4). The predicted secondary structure of GAI shows few salient features. GAI is a largely hydrophilic protein with a polyhistidine tract of unknown significance close to the
15 amino-terminus, and a weakly hydrophobic domain surrounding a possible glycosylation site at Asn¹⁸³. Computer analysis indicates a relatively low likelihood that this hydrophobic region is a transmembrane domain.

Searches of the DNA and protein sequence databases
20 revealed no domains of obvious functional significance within GAI. *gai* contains a deletion of 51 bp from within the GAI ORF. This in-frame deletion results in the absence, in *gai*, of a 17 amino acid residue segment situated close to the amino terminus of the predicted
25 GAI protein (Figure 4).

Laurenzio et al.⁴⁵ reported after the priority date of the present invention a sequence for the SCR (SCARECROW) gene of *Arabidopsis*, mutation of which

results in roots that are missing one cell layer. The disclosed SCR sequence has some homology with the *Arabidopsis* GAI sequence of the present invention, but lacks the 17 amino acid motif discussed.

5 A previous publication described the isolation, following γ -irradiation mutagenesis, of *gai* derivative alleles⁵. These alleles, when homozygous, confer a tall phenotype indistinguishable from that conferred by GAI⁵. Sequencing of amplified fragments from several of the
10 derivative alleles (*gai-d1*, *gai-d2*, *gai-d5* and *gai-d7*) showed that each contains the 51 bp deletion characteristic of *gai*. Nucleotide and encoded amino acid sequences of these alleles are shown in Figure 6. They also contain additional mutations that could confer
15 a non-functional gene product (Table 1). The fact that loss of *gai* mutant phenotype is correlated with each of these mutations, together with the reversion data (see above), confirms that GAI has been cloned. Furthermore, these results are consistent with predictions that the
20 *gai-d* alleles would be null alleles^{5,6}.

Cloning of *gai* via insertional mutagenesis was possible because it is a gain-of-function mutation. Such mutations can have dominant effects for a variety of reasons, including ectopic or increased expression of
25 a normal gene product, or altered function of a mutant gene product. Here we show that the *gai* mutation is associated with an altered product. Deletion of a 17 amino acid residue domain from GAI results in a mutant

protein (*gai*) which, in a genetically dominant fashion, causes dwarfism. This strongly suggests that GAI is a growth repressor, and that GA de-represses growth by antagonizing GAI action. The domain missing in the mutant *gai* protein may be responsible for interacting with the GA signal or with GA itself. *gai* would then constitutively repress growth because it cannot be antagonized by GA. A de-repression model for GA-mediated plant growth regulation is further elaborated in Figure 5, but it should be noted that this proposal is not to be taken to limit the scope of the present invention. Knowledge of the actual mode of action of GAI and *gai*, i.e. how they work, is not a pre-requisite for operation of the present invention, which is founded on cloning of wild-type and mutant versions of the GAI gene.

Mutations at the *SPINDLY* (*SPY*) locus of *Arabidopsis* confer increased resistance to GA biosynthesis inhibitors and a reduced dependence on GA for growth regulation¹⁸, phenotypes characteristic of the slender mutants previously described in other plant species¹⁹⁻²³. Recent experiments have shown that the dwarf phenotype conferred by *gai* can be partially suppressed by mutations at *SPY* and at other loci^{6,9}. We propose, again without limiting the scope of the present invention, that *SPY*, together with proteins encoded by these other loci, is involved with the downstream transduction of the growth repressing signal that

originates with GAI (Figure 5).

According to the model shown in Figure 5, GA de-represses plant growth because it (or a GA signalling component) antagonizes the activity of GAI, a protein 5 which represses growth. The growth repressing signal is transmitted via SPY^{6,18}, GAR2⁶, GAS2 (J.P. and N.P.H., unpublished) and other proteins. Normal plants (GAI) grow tall because the level of endogenous GA is sufficiently high to substantially antagonize the 10 activity of the GAI repressor. GA-deficient plants contain insufficient GA to antagonize GAI repression to the same degree, and are thus dwarfed²⁵⁻²⁷. *gai* mutant plants are dwarfed² because the mutant *gai* protein is not antagonized by GA, and represses growth in a 15 dominant fashion. *spy*, *gar2* and *gas2* mutations partially suppress *gai* phenotype, and confer resistance to GA biosynthesis inhibitors^{6,18}. Pairwise combinations of these three mutations confer more extreme *gai* suppression and resistance to GA 20 biosynthesis inhibition than is conferred by any of *spy*, *gar2* or *gas2* alone. Thus, these genes are proposed to encode downstream components that are responsible for the transmission of the growth repressing signal from GAI. It is possible that the *gai* mutation is a 25 functional homologue of the GA-insensitivity mutations in maize¹⁰⁻¹² and wheat¹³. Thus this model can be used to provide a general explanation for the regulation of plant growth by GA.

Independent studies of GA-insensitive dwarf mutants in maize^{11,12}, and GA-independent slender mutants in pea and barley¹⁹⁻²³, have previously implicated the involvement of a repressor function in GA signal-transduction. The indications from the worked described herein are that in all probability *Arabidopsis* GAI is such a repressor. An important implication of this is that GA then regulates plant growth not via activation but by de-repression.

10

EXAMPLE 2

Cloning of GAI homologues from wheat, rice and Brassica sps

DNA containing potential GAI homologues are isolated from wheat, rice and *Brassica* by reduced stringency probing of cDNA or genomic DNA libraries containing DNA from these species. Hybridizing clones are then purified using standard techniques.

Alternatively, potential GAI homologues are identified by screening of EST databases for cDNA and other sequences showing statistically significant homology with the GAI sequence. Clones are then obtained by requesting them from the relevant distribution centres. Table 2 gives details of results of searching in public sequence databases containing EST sequences that were obtained in random sequencing programmes, showing that homologous sequences have been found in various species, including *Zea Mays* (maize), *O.*

Sativa (rice), and *Brassica napus* (rape).

In the case of wheat and maize, it is important to know if these homologous sequences correspond to the previously characterized *Rht* and *D8* genetic loci. This is determined as follows.

cDNA or genomic DNA from rice, wheat or maize is mapped onto the wheat genomic map, thus determining if the map position of the DNA corresponds to the map position of the *Rht* loci in wheat. Furthermore, in the case of maize, potential transposon-insertion alleles of *D8* exist, and these are used to prove the cloning of *D8* in the same manner as we have proven the cloning of *gai* from *Arabidopsis*. By sequencing these various cDNA and genomic DNA clones, studying their expression patterns and examining the effect of altering their expression, genes carrying out a similar function to *GAI* in regulating plant growth are obtained.

Mutants, derivatives, variants and alleles of these sequences are made and identified as appropriate.

20

EXAMPLE 3

Expression of *GAI* and *gai* proteins in *E. coli*

DNA fragments containing the complete *GAI* or *gai* open reading frames were amplified using PCR from genomic DNA clones (no introns in gene) containing the *GAI* and *gai* genes. Amplifications were done using primers which converted the ATG translation start codon into a *Bam*HI restriction endonuclease site. The

fragments have a PstI restriction endonuclease site at the other end (beyond the stop codon). The products were cloned and their DNA sequences determined to ensure that no errors had been introduced during the course of the PCR. The correct fragments were cloned into BamHI/PstI digested PQE30 expression vector (Qiaexpressionist kit from the Qiagen Company), resulting in constructs with the potential to express the GAI and gai proteins in *E. coli*. Expression in this vector is regulated by an IPTG-inducible promoter, and the resultant proteins carry an N-terminal polyhistidine tag which can be used to purify them from cellular extracts.

Induction with IPTG resulted in high-level expression of the GAI and gai proteins in *E. coli*.

EXAMPLE 4

Expression constructs and transformation of plants

(a) Normal expression levels, using endogenous promoters

The GAI and gai genes were isolated as 5kb EcoRI/EcoRV fragments (containing about 1.5kb of non-coding sequence flanking the coding sequence) by subcloning from appropriate genomic clones. These fragments were cloned into the Bluescript vector, re-isolated as EcoRI/XbaI fragments, and ligated into binary vectors for mobilisation into *Agrobacterium tumefaciens* C58C1, with the T-DNA being introduced into *Arabidopsis* and tobacco plants as described by Valvekens

et al.³² or by the more recent vacuum infiltration method³³, and into *Brassica napus* using the high efficiency *Agrobacterium* transformation technique as described in Moloney et al.³⁴.

5

(b) Overexpression using an exogenous promoter

Constructs have been made using DNA from vectors pJIT60, containing a double 35S promoter³⁵ and pJIT62, a modified form of pJIT60 that contains a single 35S
10 promoter. The promoters from these vectors were fused with around 100bp 5' non-coding sequence, followed by an ATG and the entire *GAI* or *gai* open reading frames, followed by a translational stop codon, followed by around 20bp 3' non-coding sequence, followed by a
15 polyadenylation signal: all this carried on a *Sst*I/*Xho*I fragment.

This fragment has been ligated into binary vectors for introduction into transgenic plants, either by the use of *Agrobacterium tumefaciens* or as naked DNA, as
20 described earlier.

EXAMPLE 4

Modification of GAI and gai sequences

A short segment of the *GAI* open reading frame
25 surrounding the *gai* deletion is amplified from *GAI* and *gai* by using in PCR appropriate oligonucleotide primers, designed on the basis of sequence information provided herein. The amplified segment is then subjected to onr

or more of various forms of mutagenesis (see e.g. Sambrook et al.), resulting in a series of overlapping deletion mutants, or, if desired, substitutions of individual nucleotides in this region.

5 The mutated amplified segment is then substituted for the equivalent segment in *GAI*, via restriction endonuclease digestion and a subsequent ligation reaction. This new variant is then expressed in transgenic plants either at normal levels or via
10 overexpression as described above.

Constructs are studied to assess their effects on plant growth regulation in model (e.g. *Arabidopsis* and tobacco) and crop (e.g. wheat, rice and maize) species. Different constructs confer differing degrees of
15 dwarfism and may individually be especially suited to the modification and improvement of particular crop species, or for crops growing in particular environments.

20 *EXAMPLE 5*

GAI null alleles confer increased resistance to paclobutrazol:

Paclobutrazol is a triazole derivative that specifically inhibits GA biosynthesis at the kaurene
25 oxidase reaction^{36,37}, thus reducing endogenous GA levels and conferring a dwarf phenotype on plants exposed to it. The slender mutants of pea and barley are resistant to the dwarfing effects of paclobutrazol³⁸⁻⁴², as is the

Arabidopsis constitutive GA-response mutant *spy*^{43,44}. Thus, in these mutants stem elongation is at least partially uncoupled from the GA-mediated control characteristic of normal plants. Interestingly, the
5 *gai-t6* mutant also displays paclobutrazol resistance. When grown on medium containing paclobutrazol, *gai-t6* mutants display longer floral bolt stems than *GAI* control plants. This result suggests that loss of *GAI* function causes a reduction in the GA-dependency of stem
10 elongation. Put another way, a *GAI* null mutant appears to require less endogenous GA to achieve a certain degree of growth than does a normal plant. GA-dependency is not completely abolished by *gai-t6* possibly because the products of genes related in
15 sequence to *GAI* (see above) can substantially, but not completely, compensate for loss of *GAI* function. These observations are significant, because they demonstrate that the wild-type gene product, *GAI*, is a GA signal-transduction component.

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TABLE 1 Mutations in *GAI* alleles

Allele	Nature of Mutation*	Position in Coding Sequence	Consequence of Mutation
<i>gai-d1</i>	<u>C</u> AG to <u>T</u> AG	Glu ²³⁹	Stop codon, truncated polypeptide
<i>gai-d2</i>	GAT to GA, one base deletion aa?> p+Á ■t? ■■	Asp ²⁷⁴	Frameshift, addition of two novel amino acids, truncated polypeptide
<i>gai-d5</i>	7 base deletion, also <u>C</u> to <u>G</u>	follows Leu ²⁸¹	Frameshift, addition of 18 novel amino acids, truncated polypeptide
<i>gai-d7</i>	GTT to GT, one base deletion	Val ¹⁵⁶	Frameshift, addition of 27 novel amino acids, truncated polypeptide

*Underlining denotes nucleotide substitution in each allele. The alleles were isolated following γ -irradiation mutagenesis of *gai* homozygotes⁵. 1.7 kb fragments were amplified from genomic DNA from each allele, and sequenced as described above. Each allele contains the 51 bp deletion characteristic of *gai*, confirming that they are all genuinely derived from *gai* and are not contaminants.

Databases searched on 11/1/96

Table 2ESTs with homology to the GAI c-DNA1.- HOMOLOGY TO THE FIRST 200 AMINOACIDS.

<u>Clone ID</u>	<u>Species</u>	<u>Blast Poisson probability</u>
EM_EST1:ATTS3217	A.Thaliana	4.8 . e ⁻³²
EM_EST1:AT7823	A.Thaliana	4.8 . e ⁻²⁴
EM_EST1:AT7938	A.Thaliana	7.2 . e ⁻²²
EM_EST3:OSS0803A	O.Sativa (rice)	7.8 . e ⁻¹¹
EM_EST1:AT5178	A.Thaliana	0.014
EM_EST1:AT9456	A.Thaliana	0.026

2.- HOMOLOGY TO AMINOACIDS 200-400.

<u>Clone ID</u>	<u>Species</u>	<u>Blast Poisson probability</u>
EM_EST1:ATTS4818	A.Thaliana	1.5 . e ⁻²¹
EM_EST3:ZM3101	Zea Mays (maize)	9.1 . e ⁻¹⁴
EM_EST1:ATTS1110	A.Thaliana	7.9 . e ⁻¹⁰
EM_EST1:ATTS3935	A.Thaliana	1.7 . e ⁻⁹
EM_ST3:ZM7862	Zea Mays (maize)	4.5 . e ⁻⁷
EM_EST1:AT7938	A.Thaliana	0.00011
EM_EST3:OSS3989A	O.Sativa (rice)	0.00050

3.- HOMOLOGY TO THE LAST 132 AMINOACIDS.

<u>Clone ID</u>	<u>Species</u>	<u>Blast Poisson probability</u>
EM_EST1:AT2057	A.Thaliana	3.1 . e ⁻⁵²
EM_EST1:ATTS3359	A.Thaliana	3.2 . e ⁻⁴²
EM_EST3:OSO713A	O.Sativa (rice)	2.8 . e ⁻¹⁰
EM_EST1:BN6691	B. Napus (rape)	3.0 . e ⁻⁵
EM_EST1:ATTS3934	A.Thaliana	0.00034
EM_EST1:ATTS4819	A.Thaliana	0.00059
EM_EST1:AT4839	A.Thaliana	0.00060
EM_EST1:ATTS1327	A.Thaliana	0.00073
EM_EST1:AT1868	A.Thaliana	0.0054
EM_EST1:AT79316	A.Thaliana	0.092
EM_EST1:AT7747	A.Thaliana	0.35

CLAIMS:

1. A nucleic acid isolate having a nucleotide sequence coding for a polypeptide which includes the amino acid sequence shown in Figure 4.
- 5 2. Nucleic acid according to claim 1 wherein the coding nucleotide sequence includes the coding nucleotide sequence shown in Figure 3.
- 10 3. Nucleic acid according to claim 1 wherein the coding nucleotide sequence includes a mutant, allele, derivative or variant, by way of addition, substitution, insertion and/or deletion of one or more nucleotides, of the coding nucleotide sequence shown
15 in Figure 3.
4. A nucleic acid isolate having a nucleotide sequence coding for a polypeptide which includes an amino acid sequence which is a mutant, allele,
20 derivative or variant sequence of the GAI amino acid sequence of the species *Arabidopsis thaliana* shown in Figure 4, or is a homologue of another species or a mutant, allele, derivative or variant thereof, wherein said mutant, allele, derivative, variant or homologue
25 differs from the amino acid sequence shown in Figure 4 by way of insertion, deletion, addition and/or substitution of one or more amino acids, wherein expression of said nucleic acid in a plant results in

inhibition of growth of the plant, the inhibition being antagonised by gibberellin (GA).

5. Nucleic acid according to claim 4 wherein over-
5 expression of said nucleic acid in a plant confers a dwarf phenotype on the plant, which dwarf phenotype is correctable by treatment with GA.
6. Nucleic acid according to claim 4 or claim 5
10 wherein said polypeptide includes the 17 amino acid sequence underlined in Figure 4.
7. Nucleic acid according to claim 4 or claim 5
15 wherein said polypeptide includes a contiguous sequence of 17 amino acid residues in which at least 10 residues have similarity with a residue in the corresponding position in the 17 amino acid sequence underlined in Figure 4.
- 20 8. A nucleic acid isolate having a nucleotide sequence coding for a polypeptide which includes an amino acid sequence which is a mutant, allele, derivative or variant sequence of the GAI amino acid sequence of the species *Arabidopsis thaliana* shown in
25 Figure 4, or is a homologue of another species or a mutant, allele, derivative or variant thereof, wherein said mutant, allele, derivative, variant or homologue differs from the amino acid sequence shown in Figure 4

by way of insertion, deletion, addition and/or substitution of one or more amino acids, wherein expression of said nucleic acid complements a GAI null mutant phenotype in a plant, such phenotype being
5 resistance to the dwarfing effect of paclobutrazol.

9. Nucleic acid according to any of claims 4 to 8 wherein said plant is *Arabidopsis thaliana*.

10 10. A nucleic acid isolate having a nucleotide sequence coding for a polypeptide which includes the amino acid sequence encoded by nucleic acid according to claim 8 save for deletion of the 17 amino acid sequence underlined in Figure 4 or a contiguous 17
15 amino acid sequence in which at least 10 residues have similarity with a residue in the corresponding position in the 17 amino acid sequence underlined in Figure 4.

20 11. A nucleic acid isolate having a nucleotide sequence coding for a polypeptide which includes an amino acid sequence which is a mutant, allele, derivative or variant sequence, by way of insertion, deletion, addition and/or substitution of one or more
25 amino acids, of the GAI amino acid sequence of the species *Arabidopsis thaliana* shown in Figure 4 or a homologue of another species, wherein expression of said nucleic acid in a plant confers a phenotype on

the plant which is gibberellin-unresponsive dwarfism.

12. Nucleic acid according to claim 11 wherein the polypeptide includes the amino acid sequence shown in
5 Figure 4 with the underlined 17 amino acids deleted.

13. Nucleic acid according to claim 12 wherein the coding nucleotide sequence includes the coding nucleotide sequence shown in Figure 3 but with the
10 nucleotides which encode the amino acids underlined in Figure 4 deleted.

14. Nucleic acid according to claim 12 wherein the coding nucleotide sequence includes a nucleotide
15 sequence which is a mutant, allele, derivative or variant sequence, by way of insertion, deletion, addition and/or substitution of one or more nucleotides, of the nucleotide sequence shown in Figure 3 but with the nucleotides which encode the
20 amino acids underlined in Figure 4 deleted.

15. Nucleic acid according to claim 11 wherein the polypeptide has an amino acid sequence which is a mutant, allele, derivative or variant sequence of the
25 amino acid sequence shown in Figure 4 by way of deletion of the 17 amino acids underlined in Figure 4 and the addition, insertion, substitution and/or deletion of one or more amino acids.

16. Nucleic acid according to any of claims 11 to 15 wherein said plant is *Arabidopsis thaliana*.
17. A nucleic acid having a nucleotide sequence
5 coding for a polypeptide which includes an amino acid sequence which is a mutant, allele, derivative or variant sequence, by way of insertion, deletion, addition and/or substitution of one or more amino acids, of the GAI amino acid sequence of the species
10 *Arabidopsis thaliana* shown in Figure 4, wherein the polypeptide has the amino acid sequence shown in Figure 6b, Figure 6d, Figure 6f or Figure 6h.
18. Nucleic acid according to claim 17 wherein the
15 coding nucleotide sequence is that shown in Figure 6a, Figure 6c, Figure 6e or Figure 6g.
19. Nucleic acid according to any of claims 1 to 18 further including a regulatory sequence for expression
20 from said coding nucleotide sequence.
20. Nucleic acid according to claim 19 wherein the regulatory sequence includes an inducible promoter.
- 25 21. A nucleic acid isolate having a nucleotide sequence complementary to a sequence of at least 14 contiguous nucleotides of the coding sequence or sequence complementary to the coding sequence of

nucleic acid according to any of claims 1 to 15
suitable for use in anti-sense or sense regulation
("co-suppression") of expression said coding sequence.

- 5 22. Nucleic acid according to claim 21 which is DNA
and wherein said complementary nucleotide sequence is
under control of a regulatory sequence for anti-sense
transcription.
- 10 23. Nucleic acid according to claim 22 wherein the
regulatory sequence includes an inducible promoter.
24. A nucleic acid vector suitable for transformation
of a plant cell and including nucleic acid according
15 to any preceding claim.
25. A host cell containing heterologous nucleic acid
according to any preceding claim.
- 20 26. A host cell according to claim 25 which is
microbial.
27. A host cell according to claim 25 which is a
plant cell.
- 25 28. A plant cell according to claim 27 having
heterologous said nucleic acid within its genome.

29. A plant cell according to claim 28 having more than one said nucleotide sequence per haploid genome.
30. A plant cell according to any of claims 27 to 29
5 which is comprised in a plant, a plant part or a plant propagule, or an extract or derivative of a plant.
31. A method of producing a cell according to any of claims 25 to 30, the method including incorporating
10 said nucleic acid into the cell by means of transformation.
32. A method according to claim 31 which includes recombining the nucleic acid with the cell genome
15 nucleic acid such that it is stably incorporated therein.
33. A method according to claim 31 or claim 32 which includes regenerating a plant from one or more
20 transformed cells.
34. A plant comprising a plant cell according to any of claims 27 to 29.
- 25 35. A plant which is a sexually or asexually propagated off-spring, clone or descendant of a plant according to claim 31, or any part or propagule of said plant, off-spring, clone or descendant.

36. A part or propagule, or extract or derivative of a plant according to claim 35.

37. A method of producing a plant, the method
5 including incorporating nucleic acid according to any of claims 1 to 24 into a plant cell and regenerating a plant from said plant cell.

38. A method according to claim 37 including sexually
10 or asexually propagating or growing off-spring or a descendant of the plant regenerated from said plant cell.

39. A method of influencing a characteristic of a
15 plant, the method including causing or allowing expression from heterologous nucleic acid according to any of claims 1 to 3 within cells of the plant.

40. A method of influencing a characteristic of a
20 plant, the method including causing or allowing expression of from heterologous nucleic acid according to any of claims 4 to 7 within cells of the plant.

41. A method of influencing a characteristic of a
25 plant, the method including causing or allowing expression of from heterologous nucleic acid according to claim 8 or claim 9 within cells of the plant.

42. A method of influencing a characteristic of a plant, the method including causing or allowing expression of from heterologous nucleic acid according to any of claims 10 to 16 within cells of the plant.

5

43. A method of influencing a characteristic of a plant, the method including causing or allowing transcription from nucleic acid according to any of claims 21 to 23 within cells of the plant.

10

44. Use of nucleic acid according to any of claims 1 to 3 in the production of a transgenic plant.

45. Use of nucleic acid according to any of claims 4 to 7 in the production of a transgenic plant.

15

46. Use of nucleic acid according to claim 8 or claim 9 in the production of a transgenic plant.

47. Use of nucleic acid according to any of claims 10 to 16 in the production of a transgenic plant.

20

48. Use of nucleic acid according to any of claims 21 to 23 in the production of a transgenic plant.

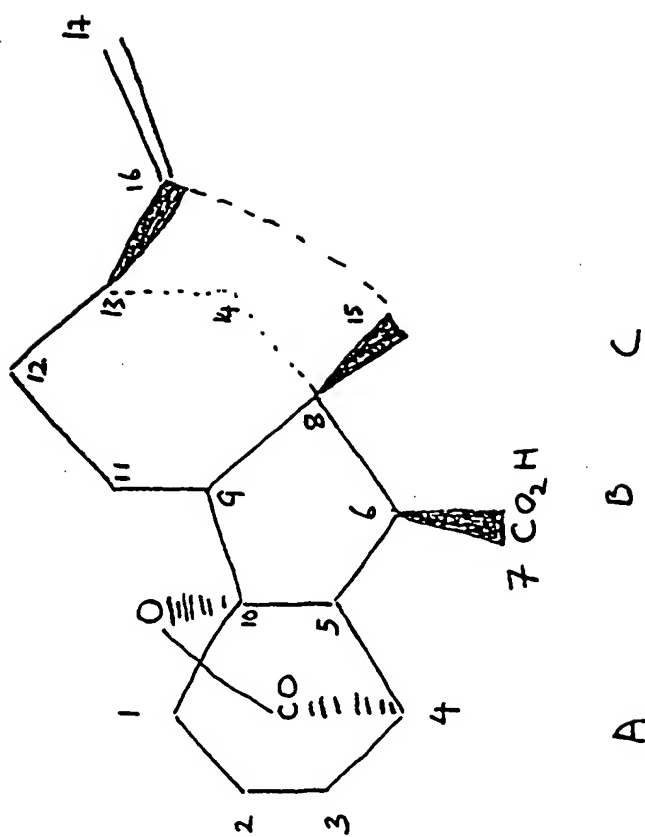


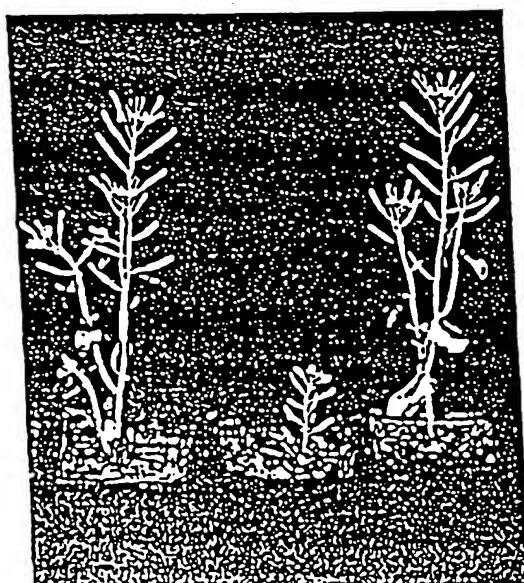
Fig. 1

Fig. 2 b

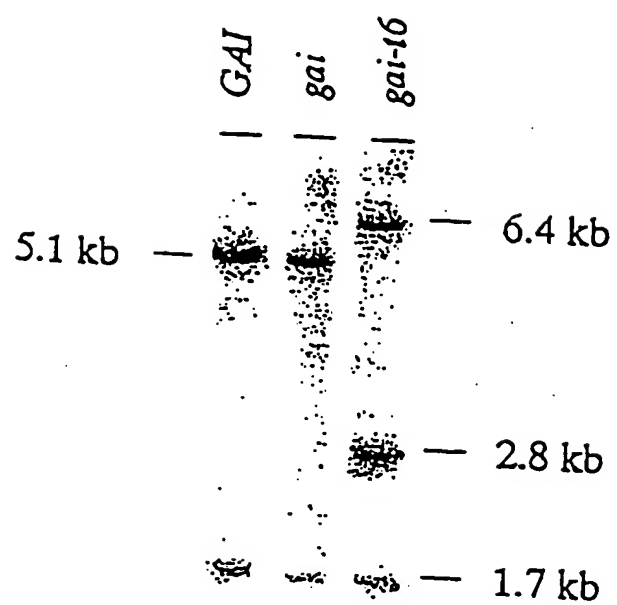
GAI
—
gai
—
gai-t6
—

— 18 kb
— 15.5 kb

Fig. 2 a



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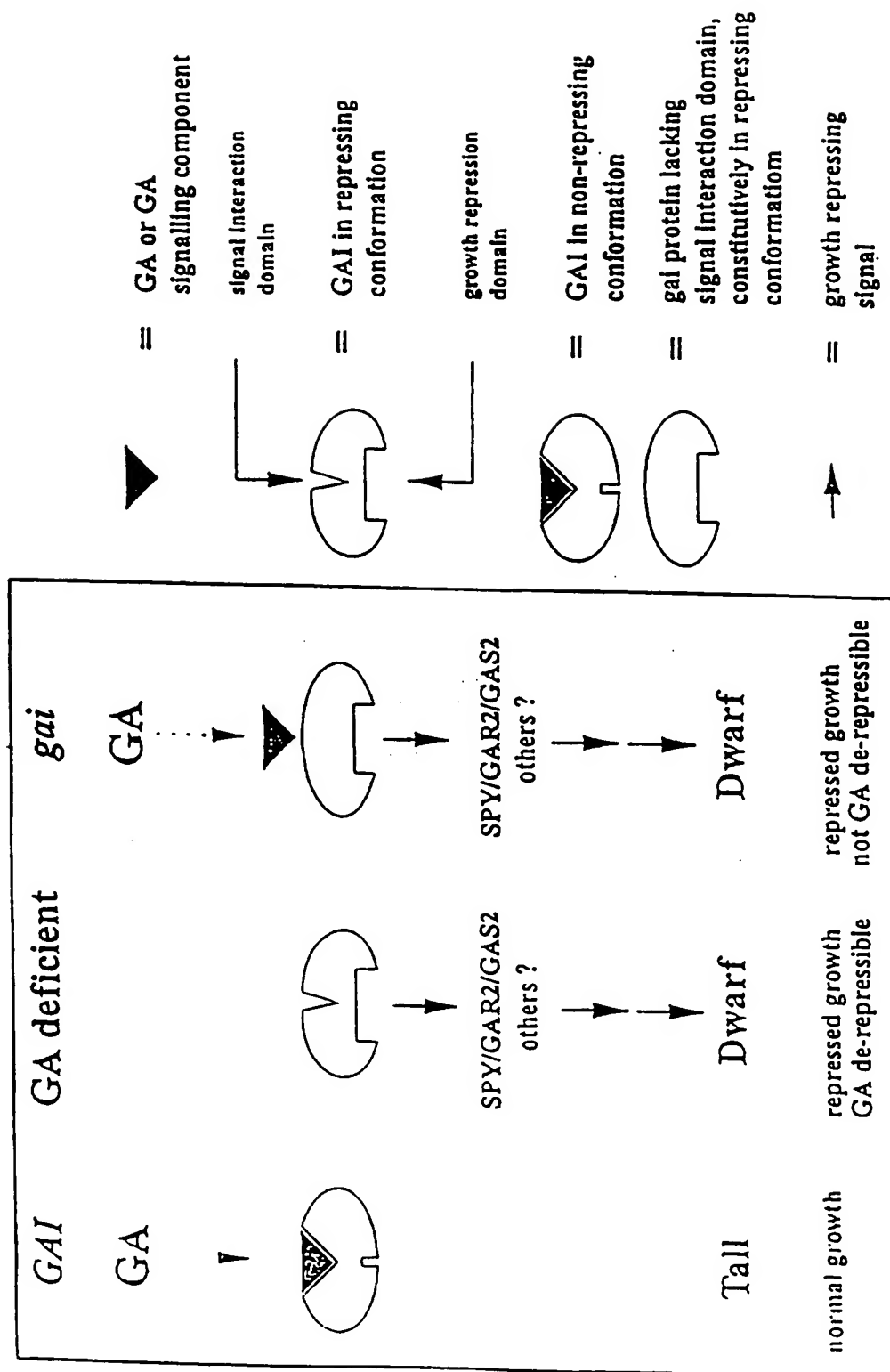
Fig. 2c

TAATAATCAT	TTTTTTTCTT	ATAACCTTCC	TCTCTATTTT	TACAATTTAT	TTTGTTATTA	60
GAAGTGGTAG	TGGAGTGAAA	AAACAAATCC	TAAGCAGTCC	TAACCGATCC	CCGAAGCTAA	120
AGATTCTTCA	CCTTCCCAAA	TAAAGCAAAA	CCTAGATCCG	ACATTGAAGG	AAAAACCTTT	180
TAGATCCATC	TCTGAAAAAA	AACCAACCAT	GAAGAGAGAT	CATCATCATC	ATCATCAAGA	240
TAAGAAGACT	ATGATGATGA	ATGAAGAAGA	CGACGGTAAC	GGCATGGATG	AGCTTCTAGC	300
TGTTCTTGGT	TACAAGGTTA	GGTCATCGGA	AATGGCTGAT	GTTGCTCAGA	AACTCGAGCA	360
GCTTGAAGTT	ATGATGTCTA	ATGTTCAAGA	AGACGATCTT	TCTCAACTCG	CTACTGAGAC	420
TGTTCACTAT	AATCCGGCGG	AGCTTTACAC	GTGGCTTGAT	TCTATGCTCA	CCGACCTTAA	480
TCCTCCGTCG	TCTAACGCCG	AGTACGATCT	TAAAGCTATT	CCCGGTGACG	CGATTCTCAA	540
TCAGTTCGCT	ATCGATTCCG	CTTCTTCGTC	TAACCAAGGC	GGCGGAGGAG	ATACGTATAC	600
TACAAACAAG	CGGTTGAAAT	GCTCAAACGG	CGTCGTGGAA	ACCACCACAG	CGACGGCTGA	660
GTCAACTCGG	CATGTTGTCC	TGGTTGACTC	GCAGGAGAAC	GGTGTGCGTC	TCGTTACGCG	720
GCTTTTGGCT	TGCGCTGAAG	CTGTTTCAGAA	GGAGAATCTG	ACTGTGGCGG	AAGCTCTGGT	780
GAAGCAAATC	GGATTCTTAG	CTGTTTCTCA	AATCGGAGCT	ATGAGAAAAG	TCGCTACTTA	840
CTTCCGCCGA	GCTCTCGCGC	GGCGGATTTA	CCGTCTCTCT	CCGTCCGAGA	GTCCAATCGA	900
CCACTCTCTC	TCCGATACTC	TTCAGATGCA	CTTCTACGAG	ACTTGTCTTT	ATCTCAAGTT	960
CGCTCACTTC	ACGGCGAATC	AAGCGATTCT	CGAAGCTTTT	CAAGGGAAGA	AAAGAGTTCA	1020
TGTCATTGAT	TTCTCTATGA	GTCAAGGTCT	TCAATGGCCG	GCGCTTATGC	AGGCTCTTGC	1080
GCTTCGACCT	GGTGGTCTCT	CTGTTTTCCG	GTTAACCAGG	ATTGGTCCAC	CGGCACCGGA	1140
TAATTTTCGAT	TATCTTCATG	AAGTTGGGTG	TAAGCTGGCT	CATTTAGCTG	AGGCGATTCA	1200
CGTTGAGTTT	GAGTACAGAG	GATTTGTGGC	TAACACTTTA	GCTGATCTTG	ATGCTTCGAT	1260
GCTTGAGCTT	AGACCAAGTG	AGATTGAATC	TGTTGCGGTT	AACTCTGTTT	TCGAGCTTCA	1320
CAAGCTCTTG	GGACGACCTG	GTGCGATCGA	TAAGGTTCTT	GGTGTGGTGA	ATCAGATTAA	1380
ACCGGAGATT	TTCAGTGTGG	TTGAGCAGGA	ATCGAACCAT	AATAGTCCGA	TTTTCTTAGA	1440
TOGGTTTACT	GAGTCGTTGC	ATTATTACTC	GACGTTGTTT	GACTCGTTGG	AAGGTGTACC	1500
GAGTGGTCAA	GACAAGGTCA	TGTCGGAGGT	TTACTTGGGT	AAACAGATCT	GCAACGTTGT	1560
GGCTTGTGAT	GGACCTGACC	GAGTTGAGCG	TCATGAAACG	TTGAGTCAGT	GGAGGAACCG	1620
GTTCCGGTCT	GCTGGGTTTG	CGGCTGCACA	TATTGGTTTCG	AATGCGTTTA	AGCAAGCCAG	1680
TATGCTTTTG	GCTCTGTTCA	ACGGCGGTGA	GGGTTATCGG	GTGGAGGAGA	GTGACGGCTG	1740
TCTCATGTTG	GGTTGGCACA	CACGACCGCT	CATAGCCACC	TCGGCTTGGA	AACTCTCCAC	1800
CAATTAGATG	GTGGCTCAAT	GAATTGATCT	GTTGAACCGG	TTATGATGAT	AGATTTCCGA	1860
CCGAAGCCAA	ACTAAATCCT	ACTGTTTTTC	CCTTTGTCAC	TTGTTAAGAT	CTTATCTTTC	1920
ATTATATTAG	GTAATTGAAA	AATTTCTAAA	TTACTCACAC	TGGC		1964

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MetLysArgAspHisHisHisHisHisGlnAspLysLysThrMetMetMetAsnGluGlu	20
AspAspGlyAsnGlyMet <u>AspGluLeuLeuAlaValLeuGlyTyrLysValArgSerSer</u>	40
<u>GluMetAla</u> AspValAlaGlnLysLeuGluGlnLeuGluValMetMetSerAsnValGln	60
GluAspAspLeuSerGlnLeuAlaThrGluThrValHisTyrAsnProAlaGluLeuTyr	80
ThrTrpLeuAspSerMetLeuThrAspLeuAsnProProSerSerAsnAlaGluTyrAsp	100
LeuLysAlaIleProGlyAspAlaIleLeuAsnGlnPheAlaIleAspSerAlaSerSer	120
SerAsnGlnGlyGlyGlyGlyAspThrTyrThrThrAsnLysArgLeuLysCysSerAsn	140
GlyValValGluThrThrThrAlaThrAlaGluSerThrArgHisValValLeuValAsp	160
SerGlnGluAsnGlyValArgLeuValHisAlaLeuLeuAlaCysAlaGluAlaValGln	180
LysGluAsnLeuThrValAlaGluAlaLeuValLysGlnIleGlyPheLeuAlaValSer	200
GlnIleGlyAlaMetArgLysValAlaThrTyrPheAlaGluAlaLeuAlaArgArgIle	220
TyrArgLeuSerProSerGlnSerProIleAspHisSerLeuSerAspThrLeuGlnMet	240
HisPheTyrGluThrCysProTyrLeuLysPheAlaHisPheThrAlaAsnGlnAlaIle	260
LeuGluAlaPheGlnGlyLysLysArgValHisValIleAspPheSerMetSerGlnGly	280
LeuGlnTrpProAlaLeuMetGlnAlaLeuAlaLeuArgProGlyGlyProProValPhe	300
ArgLeuThrGlyIleGlyProProAlaProAspAsnPheAspTyrLeuHisGluValGly	320
CysLysLeuAlaHisLeuAlaGluAlaIleHisValGluPheGluTyrArgGlyPheVal	340
AlaAsnThrLeuAlaAspLeuAspAlaSerMetLeuGluLeuArgProSerGluIleGlu	360
SerValAlaValAsnSerValPheGluLeuHisLysLeuLeuGlyArgProGlyAlaIle	380
AspLysValLeuGlyValValAsnGlnIleLysProGluIlePheThrValValGluGln	400
GluSerAsnHisAsnSerProIlePheLeuAspArgPheThrGluSerLeuHisTyrTyr	420
SerThrLeuPheAspSerLeuGluGlyValProSerGlyGlnAspLysValMetSerGlu	440
ValTyrLeuGlyLysGlnIleCysAsnValValAlaCysAspGlyProAspArgValGlu	460
ArgHisGluThrLeuSerGlnTrpArgAsnArgPheGlySerAlaGlyPheAlaAlaAla	480
HisIleGlySerAsnAlaPheLysGlnAlaSerMetLeuLeuAlaLeuPheAsnGlyGly	500
GluGlyTyrArgValGluGluSerAspGlyCysLeuMetLeuGlyTrpHisThrArgPro	520
LeuIleAlaThrSerAlaTrpLysLeuSerThrAsn	532

Fig. 4



FIR. 5

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Figure 6(a)

1 TAGAAGTGGT AGTCGAGTGA AAAAACAART CCTAAGCAGT CCTAACCGAT
51 CCCCGAAGCT AAAGATTCTT CACCTTCCCA AATAAAGCAA AACCTAGATC
101 CGACATTGAA GGAAAAACCT TTTAGATCCA TCTCTGAAAA AAAACCAACC
151 ATGAAGAGAG ATCATCATCA TCATCATCAA GATAAGAAGA CTATGATGAT
201 GAATGAAGAA GACGACGGTA ACGGCATGGA TGTGCTCAG AAACCTCGAGC
251 AGCTTGAAGT TATGATGTCT AATGTTCAAG AAGACGATCT TTCTCAACTC
301 GCTACTGAGA CTGTTCACTA TAATCCGGCG GAGCTTTACA CGTGGCTTGA
351 TTCTATGCTC ACCGACCTTA ATCTCCGTC GTCTAACGCC GAGTACGATC
401 TTAAAGCTAT TCCCGGTGAC GCGATTCTCA ATCAGTTCCG TATCGATTCC
451 GCTTCTTCGT CTAACCAAGG CGCGGAGGA GATACGTATA CTACAAACAA
501 GCGGTTGAAA TGCTCAAACG GCGTCGTGGA AACCACCACA GCGACGGCTC
551 AGTCAACTCG GCATGTTGTC CTGGTTCAGT CGCAGGAGAA CGGTGTGCGT
601 CTCGTTACAG CGCTTTTGGC TTCCGCTGAA GCTGTTTCTC AGGAGAATCT
651 GACTGTGGCG GAAGCTCTGG TGAAGCAAAT CCGATTCTTA GCTGTTTCTC
701 AAATCCGAGC TATGAGAAAA GTCGCTACTT ACTTCGCCGA AGCTCTCGCG
751 CGGCGGATTT ACCGTCTCTC TCCGTCGAG AGTCCAATCG ACCACTCTCT
801 CTCCGATACT CTTTAGATGC ACTTCTACGA GACTTGTCCT TATCTCAAGT
851 TCGCTCACTT CACGGCGAAT CAAGCGATC TCGAAGCTTT TCAAGGGAAG
901 AAAAGAGTTC ATGTCATTGA TTTCTCTATG AGTCAAGGTC TTCAATGGCC
951 GGCGCTTATG CAGGCTCTTG CGCTTCGACC TGGTGGTCTT CCTGTTTTCC
1001 GGTAAACCGG AATTGCTCCA CCGGCACCGG ATAATTTCTA TTATCTTCAT
1051 GAAGTTGGGT CTAAGCTGGC TCATTTAGCT GAGGCGATC ACCTTGAGTT
1101 TGAGTACAGA GGATTTGTGG CTAACACTTT AGCTGATCTT GATGCTTCGA
1151 TGCTTGAGCT TAGACCAAGT GAGATTGAAT CTGTTGCGGT TAACTCTGTT
1201 TTCGAGCTTC ACAAGCTCTT GGGACGACCT GGTGCGATCG ATAAGGTTCT
1251 TCGTGTGGTG AATCAGATTA AACCGGAGAT TTTCACTCTC GTTGAGCAGG
1301 AATCGAACCA TAATAGTCCG ATTTTCTTAG ATCGGTTTAC TGAGTCGTTG
1351 CATTATTACT CGACGTTGTT TGACTCGTTG GAAGCTGTAC CGAGTCGTCA
1401 AGACAAGGTC ATGTCGGAGG TTTACTTGGG TAAACAGATC TGCAACGTTG
1451 TGCCTTGTGA TGGACCTGAC CGAGTTCAGC GTCATGAAAC GTTGAGTCAG
1501 TGGAGGAACC GGTTCGGGTC TGCTGGGTTT GCGGCTCCAC ATATTCCCTC
1551 GAATGCCGTTT AAGCAAGCCA GTATGCTTTT GGCTCTGTTC AACGGCGGTG
1601 AGGGTTATCG GCTGGAGGAG AGTGACGGCT GTCTCATGTT CCG

Figure 6(b)

1 MKRDHHEHEQ DKKTMMNNEE DDGNGMDVAQ KLEQLVMMMS NVQEDDLSQL
51 ATETVHYNPA ELYTWLDSML TDLNPPSSNA EYDLKAIPGD AILNQFAIDS
101 ASSSNQGGGG DTYTTNKRLK CSNGVVETTT ATAESTRHVV LVDSQENGVR
151 LVHALLACAE AVQKENLTVA EALVKQIGFL AVSQIGAMRK VATYFAEALA
201 RRTYRLSPSQ SPIDHSLSDT L*

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Figure 6(c)

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1  TAGAAGTGGT AGTGGAGTCA AAAAACAAAT CCTAAGCAGT CCTAACCGAT
51  CCCCGAAGCT AAAGATTCTT CACCTTCCCA AATAAAGCAA AACCTAGATC
101 CGACATTGAA GGAAAAACCT TTTAGATCCA TCTCTGAAAA AAAACCAACC
151 ATGAAGAGAG ATCATCATCA TCATCATCAA GATAAGAAGA CTATGATGAT
201 GAATGAAGAA GACGACGGTA ACGGCATGGA TGTTCGTCAG AAACTCGAGC
251 AGCTTCAAGT TATGATGTCT AATGTTCAAG AAGACGATCT TTCTCAACTC
301 GCTACTGAGA CTGTTCACTA TAATCCGGCG GAGCTTTACA CGTGGCTTGA
351 TTCTATGCTC ACCGACCTTA ATCCTCCGTC GTCTAACGCC GAGTACGATC
401 TTAAAGCTAT TCCCGGTGAC GCGATTCTCA ATCAGTTCCG TATCGATTCC
451 GCTTCTTCGT CTAACCAAGG CGCGGGAGGA CATACGTATA CTACAAACAA
501 GCGGTTGAAA TGCTCAAACG GCGTCGTGGA AACCACCACA GCGACGGCTG
551 AGTCAACTCC GCATGTTGTC CTGGTTGACT CGCAGGAGAA CCGTGTGCGT
601 CTCGTTACCG CGCTTTTGGC TTGCGCTGAA GCTGTTTACA AGGAGAATCT
651 GACTGTGGCG GAAGCTCTGG TGAAGCAAAT CGGATTCTTA GCTGTTTCTC
701 AAATCGGAGC TATGAGAAAA GTCGCTACTT ACTTCGCCGA AGCTCTCGCG
751 CGGCGGATTT ACCGTCTCTC TCCGTGCGAG AGTCCAATCG ACCACTCTCT
801 CTCCGATACT CTTCAGATGC ACTTCTACGA GACTTGTCCT TATCTCAAGT
851 TCGCTCACTT CACGGCGAAT CAAGCGATTC TCGAAGCTTT TCAAGGGAAG
901 AAAAGAGTTC ATGTCATTGA TTCTCTATGA GTCAAGGTCT TCAATGGCCG
951 GCGCTTATGC AGGCTCTTGC GCTTCGACCT GGTGGTCTCT CTGTTTCCG
1001 GTTAACCGGA ATTGGTCCAC CGGCACCGGA TAATTTGAT TATCTTCATG
1051 AAGTTGGGTG TAAGCTGGCT CATTTAGCTG AGGCGATTCA CGTTGAGTTT
1101 GAGTACAGAG GATTTGTGCC TAACACTTTA GCTGATCTTG ATGCTTCGAT
1151 GCTTGAGCTT AGACCAAGTG AGATTGAATC TGTTCGGTT AACTCTGTTT
1201 TCGAGCTTCA CAAGCTCTTG GGACGACCTG GTGCGATCGA TAAGGTTCTT
1251 GCTCTGGTGA ATCAGATTAA ACCGGAGATT TTCACTGTGG TTGAGCAGGA
1301 ATCGAACCAT AATAGTCCGA TTTCTTAGA TCGGTTTACT GAGTCGTTGC
1351 ATTATTACTC GACGTTGTTT GACTCGTTCC AAGGTGTACC GACTGGTCAA
1401 GACAAGGTCA TGTCGGAGGT TTAATTGGGT AAACAGATCT GCAACGTTGT
1451 GGCTTGTGAT GGACCTGACC GAGTTGAGCG TCATCAAACG TTGAGTCACT
1501 GGACCAACCG GTTCGGGTCT GCTGGGTTTG CGGCTGCACA TATGGTTTCG
1551 AATGCGTTTA AGCAAGCGAG TATGCTTTTG GCTCTGTTCA ACCGCGGTGA
1601 GGGTTATCGG GTCGAGGAGA GTGACGGCTC TCTCATCTTG GG
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Figure 6(d)

1 MKRDHHHHHQ DKKTMNMNEE DDGNGMDVAQ KLEQLEVMMS NVQEDDLSQL
51 ATETVRYNPA ELYTWLDSML TDLNPPSSNA EYDLKAIPGD AILNQFAIDS
101 ASSSNQGGGG DTYTTNKRLK CSNGVVETTT ATAESTRHVV LVDSQENGVR
151 LVHALLACAE AVQKENLTVA EALVKQIGFL AVSQIGAMRK VATYFAEALA
201 RRIYRLSPSQ SPIDHSLSDT LQMHFYETCP YLXFAHFTAN QAILEAFQGK
251 KRVHVIDSL*

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Figure 6(e)

1 TAGAAGTGGT AGTGGAGTGA AAAAACAAAT CCTAAGCAGT CCTAACCGAT
51 CCCCCAAGCT AAAGATTCTT CACCTTCCCA AATAAAGCAA AACCTAGATC
101 CGACATTGAA GGAAAAACCT TTTAGATCCA TCTCTGAAAA AAAACCAACC
151 ATGAACAGAG ATCATCATCA TCATCATCAA GATAAGAAGA CTATGATGAT
201 GAATGAAGAA GACGACGGTA ACGGCATCGA TGTTGCTCAG AAACCTCGAGC
251 AGCTTGAAGT TATGATGTCT AATGTTCAAG AAGACGATCT TTCTCAACTC
301 GCTACTGAGA CTGTTCACTA TAATCCGGCG GAGCTTTACA CCGGCTTGA
351 TTCTATGCTC ACCGACCTTA ATCTCCGTC GTCTAACGCC GAGTACGATC
401 TTAAAGCTAT TCCCGGTGAC GCGATTCTCA ATCAGTTCCG TATCGATTCC
451 GCTTCTTCGT CTAACCAAGG CGGCGGAGGA GATACGTATA CTACAAACAA
501 GCGGTTGAAA TGCTCAAACG GCGTCGTGGA AACCACCACA CGGACGGCTG
551 AGTCAACTCG GCATGTTGTC CTGGTTGACT CGCAGGAGAA CGGTGTGCGT
601 CTCGTTACG CGCTTTTGGC TTGCGCTGAA GCTGTTGAGA AGGAGAATCT
651 GACTGTGGCG GAAGCTCTGG TGAAGCAAAT CGGATTCTTA GCTGTTTCTC
701 AAATCGGAGC TATGAGAAAA GTCGCTACTT ACTTCGCCGA AGCTCTCGCG
751 CGGCGGATTT ACCGTCTCTC TCCGTGCGAG AGTCCAATCG ACCACTCTCT
801 CTCCGATACT CTTGAGATGC ACTTCTACGA GACTTGTCCT TATCTCAAGT
851 TCGCTCACTT CACGGCGAAT CAAGCGATTC TCGAAGCTTT TCAAGGGAAG
901 AAAAGAGTTC ATGTCATTGA TTTCTCTATG AGTCAAGGTC TTGGCGCTT
951 ATGCAGGCTC TTGCGCTTCG ACCTGGTGCT CCTCCTGTTT TCCGGTTAAC
1001 CGGAATTGGT CCACCGGCAC CGGATAATTT CGATTATCTT CATGAAGTTG
1051 GGTGTAAGCT GGCTCATTTA GCTCAGGCGA TTCACCTTGA GTTTCAGTAC
1101 AGAGGATTTG TGGCTAACAC TTTAGCTGAT CTTGATGCTT CGATGCTTGA
1151 GCTTAGACCA AGTGAGATTG AATCTGTTGC GGTAACTCT GTTTTCGAGC
1201 TTCACAAGCT CTTGGGACGA CCTGGTCCGA TCGATAAGGT TCTTGCTGTG
1251 GTGAATCAGA TTAAACCGGA GATTTTCACT GTGGTTGAGC AGGAATCGAA
1301 CCATAATAGT CCGATTTTCT TAGATCGGTT TACTGAGTCG TTGCATTATT
1351 ACTCGACGTT GTTTGACTCG TTGGAACGTG TACCGACTCG TCAAGACAAG
1401 GTCATGTCGG AGGTTTACTT GGGTAAACAG ATCTCCAACG TTGTGGCTTG
1451 TGATGGACCT GACCGAGTTG AGCCTCATGA AACGTTGAGT CAGTGGAGGA
1501 ACCGTTTCGG GTCTGCTGGG TTTGCGGCTG CACATATTGG TTCGAATCCG
1551 TTTAAGCAAG CGAGTATGCT TTTGGCTCTG TTCAACGGCG GTGAGGGTTA
1601 TCGGCTCGAG GACAGTGACC CCGTCTCAT GTTGGG

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Figure 6(f)

1 MCRDHHHHQ DKKTMMNNEE DDGNGMDVAQ KLEQLEVMMS NVQEDDLSQL
51 ATETVHYNPA ELYTWLDSML TDLNPPSSNA EYDLKAIPGD AILNQFAIDS
101 ASSSNQGGGG DTYTTNKRLK CSNGVVETTT ATAESTRHVV LVDSQENGVR
151 LVHALLACAE AVQKENLTV AALVKQIGFL AVSQIGAMRK VATYFAEALA
201 RRIYRLSPSQ SPIDHSLSDT LQMHFYETCP YLKFAHFTAN QAILEAFQCK
251 KRVHVIDFSM SQGLGRLCRL LRFDLVVLLF SG*

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Figure 6(g)

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1  TAGAAGTGGT AGTGGAGTGA AAAAACAAT CCTAAGCAGT CCTAACCGAT
51  CCCCAGAGCT AAAGATTCTT CACCTTCCCA AATAAGCAA AACCTAGATC
101 CGACATTGAA GGAAAAACCT TTTAGATCCA TCTCTGAAAA AAACCAACC
151 ATGAAGAGAG ATCATCATCA TCATCATCAA GATAAGAAGA CTATGATGAT
201 GAATGAAGAA GACGACGGTA ACGGCATGGA TGTGCTCAG AAACCTGAGC
251 AGCTTGAACT TATGATGTCT AATGTTCAAG AAGACGATCT TTCTCAACTC
301 GCTACTGAGA CTGTTCACTA TAATCCGGCG GAGCTTTACA CGTGGCTTGA
351 TTCTATGCTC ACCGACCTTA ATCCTCCGTC GTCTAACGCC GAGTACGATC
401 TTAAAGCTAT TCCCGGTGAC GCGATTCTCA ATCAGTTGCG TATCGATTCT
451 GCTTCTTCGT CTAACCAAGG CGGCGGAGGA GATACGTATA CTACAAACAA
501 GCGGTTGAAA TGCTCAAACG GCGTCGTGGA AACCACCACA GCGACGGCTG
551 AGTCAACTCG GCATGTGTCC TGGTTGACTC GCAGGAGAAC GGTGTGCGTC
601 TCGTTCACGC GCTTTTGGCT TCGCTGAAG CTGTTGAGAA GGAGAATCTG
651 ACTGTGGCGG AAGCTCTGGT GAAGCAAATC GGATTCTTAG CTGTTCTCA
701 AATCGGAGCT ATGAGAAAAG TCGCTACTTA CTTCGCCGAA GCTCTCGGCG
751 GGCCGATTTA CCGTCTCTCT CCGTCGCAGA GTCCAATCGA CCACTCTCTC
801 TCCGATACTC TTCAGATGCA CTCTACGAG ACTTGTCCCT ATCTCAAGTT
851 CGCTCACTTC ACGGCCAATC AAGCGATTCT CGAAGCTTTT CAAGGGAAGA
901 AAAGAGTTCA TGTCATTGAT TTCTCTATGA GTCAAGGTCT TCAATGGCCG
951 GCGCTTATGC AGGCTCTTGC GCTTCGACCT GGTGGTCCCT CTGTTTCCG
1001 GTTAACCCGA ATTGGTCCAC CGGCACCCGA TAATTTGAT TATCTTCATG
1051 AAGTTGGGTG TAAGCTGGCT CATTTAGCTG AGGCGATTCA CGTTGAGTTT
1101 GAGTACAGAG GATTTGTGGC TAACACTTTA GCTGATCTTG ATGCTTCGAT
1151 GCTTGAGCTT AGACCAAGTG AGATTGAATC TGTTCGGTT AACTCTGTTT
1201 TCGAGCTTCA CAAGCTCTTG GGACGACCTG GTCCGATCGA TAAGGTTCTT
1251 GGTGTGGTGA ATCAGATTAA ACCGGAGATT TCACTCTCG TTGAGCAGGA
1301 ATCGAACCAT AATAGTCCGA TTTTCTTAGA TCGGTTTACT GAGTCGTTGC
1351 ATTATTACTC GACGTTGTTT GACTCGTTGG AAGGTGTACC GAGTGGTCAA
1401 GACAAGGTCA TGTGGAGGT TTACTTGGGT AAACAGATCT GCAACGTTGT
1451 GCCTTGTGAT GGACCTGACC GAGTTGACCG TCATGAAACG TTGAGTCAGT
1501 GGAGGAACCG GTTCGGGTCT GCTGGGTTTG CGGCTGCACA TATTGGTTCTG
1551 AATGCGTTTA AGCAAGCGAG TATGCTTTTG GCTCTGTTCA ACGCGGTGA
1601 GCGTTATCGG GTGAGGAGA GTGACGGCTG TCTCATGTTG GG

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Figure 6(h)

1 MCRDHHHHHQ DKRTMGNVEE DDGNGRIDVAQ KLEQLEVHMS NVQEDDLSQL
51 ATETVHYNPA ELYTWLDSML TDLNPPSSNA EYDLKAIPGD AILNQFAIDS
101 ASSSNQGGGG DTYTTNKRLK CSNGVVETTT ATAESTRHVS WLTRRTVCV
151 SFTRFWLALK LFRRI*